Please replace the paragraph at page 45, line 31 to page 46, line 9 with the following paragraph:

--Thus, vaccine preparations containing attenuated toxin in accordance with the present invention are useful as adjuvants with high safety. Further, vaccines using the adjuvant of the invention are not only highly safe but also are excellent vaccines having sufficient activities of enhancing immunity even when used by non-injection vaccination route, such as intranasal, percutaneous or oral ones, in which, sufficient immunity is not usually expectable with any conventional methods. In addition, as seen in the Examples, the vaccines in accordance with the present invention are excellent in inducing local immunity, cellular immunity, and such which were problems difficult to overcome with publicly known adjuvants. Moreover, it is also possible to utilize natural toxins as safe adjuvants according to the present invention. Thus, because of the simplicity, the invention is easily applicable to the vaccine as compared with approaches of generating mutant toxins.--

REMARKS

This Preliminary Amendment is submitted in advance of the first examination of the subject application. The amendments to the specification have been made solely to clarify the invention. No new matter has been entered.

Respectfully submitted,

Date: 8/7/01

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

The paragraph at page 16, lines 18 to 34 was changed by the replacement paragraph as follows:

Because a mutant toxin has a partially altered structure as compared with that of the natural molecule, it generally loses some of the original properties of the toxin as a result of such structural changes. However, there are cases in which the activity of enhancing immunity is retained. When the toxic activity of mutant toxin is sufficiently low, e.g., reduced by a factor of at least one-two thousandth relative to that of the natural one, it can be used as an attenuated toxin for the adjuvant of the present invention provided its activity of enhancing immunity [has been] is verified. Otherwise, the mutant toxin is further attenuated by using any of the above-mentioned chemical and physical treatments to give rise to a usable adjuvant. It is preferable that the mutant toxin is stable under the conditions where the toxin-containing vaccine is to be used. Further, mutants wherein the restoration of toxic activity is limited or negligible are preferable. "Restoration of toxin activity" refers to a phenomenon in which the toxic activity, which has been reduced greatly by a variety of treatments, is recovered as time progresses after the treatment.

The paragraph at page 17, lines 14 to 22 was changed by the replacement paragraph as follows:

On the other hand, it is possible to choose amino acid residues or sugar residues to be mutated and to intentionally introduce a desired mutation in regard to the recombinant mutant toxin. Specifically, such alterations include one or more alterations of amino acid residue of oligopeptide moiety, sugar residue of oligosaccharide moiety, organic acid moiety, and such in the toxin molecule. The adjuvant of the invention also includes oligopeptide fragments such as peptide fragments playing an important role in the expression of immuno-potentiating the activity of enhancing immunity.

The paragraph at page 18, line 33 to page 19, line 8 was changed by the replacement paragraph as follows:

An artificial mutant toxin can be produced by an artificial mutant strain derived from toxin-producing wild-[stain] strain. The artificial mutant strain is generated from the [wild-strain] wild-type-strain by the treatment with a mutagenizing agent, for example. Known

methods of mutagenesis include, but are not limited to, chemical treatment, using chemical agents such as N-methyl-N'-nitro-N-nitrosoguanidine, nitrosourea, nitrogen mastard, etc., and physicochemical treatments, such as ultraviolet light irradiation, radiation exposure with cobalt-60 or the like, heating, etc. Culture of artificial mutant strains, purification of toxins from the mutant strains, and verification of the activity thereof are carried out essentially in the same manner as for the wild-type strains.

The paragraph at page 21, lines 12 to 31 was changed by the replacement paragraph as follows:

The toxin is dissolved at an adequate concentration in a buffer. While the pH is adjusted to be within a range where the toxin is stable (typically, pH 5 to 8), an agent, for example, formalin, glutaraldehyde, phenol, iodine, acid anhydride, or a detergent such as bile acid with the adequate concentration (typically, 0.01 to 1.0%) is added stepwise. Then the solution is incubated at an adequate temperature (for example, 5°C). After incubation, the agent is removed by any suitable method such as dialysis. The residual toxic activity is then assayed. If desired, the treated toxin is incubated again at a temperature at which the attenuated toxin will be used practically, for example, at 37°C, in order to [secure that the toxic activity does not recover] confirm that the attenuated toxin does not revert to toxicity. The attenuated toxin of interest is thus yielded. This method is based upon the [previous] known method widely used for [the toxoid conversion] toxoid preparation, and is also suitable for large-scale production. However, it is preferable to use a method with which the toxic activity does not [recover] restore. There are some conventional methods used for this purpose. Exemplary methods include but are not limited to (1)the simultaneous addition of lysine in a quantity corresponding to the concentration of the agent for the attenuation treatment, for example, formalin, and (2) the performance of a reduction treatment \ after the attenuation.

The paragraph at page 21, line 34 to page 22, line 12 was changed by the replacement paragraph as follows:

The toxin is dissolved at an adequate concentration in a buffer. While the pH is adjusted to a pH at which toxic activity is generally lost, including an acidic pH (for example, pH 2 to 4) or alkaline pH (for example, pH 8 to 11), the solution is incubated at an adequate

temperature. Alternatively, the solution is incubated at a temperature at which the toxic activity is generally lost (for example, at a temperature of 40°C or higher). Otherwise, the toxin is sonicated at an adequate wavelength, or irradiated with electromagnetic wave. The residual toxic activity of the sample is assayed before, during and after the treatment. If desired, the attenuated toxin is re-incubated at an adequate temperature, for example, at 37°C, [secure that the toxic activity does not recover] confirm that the attenuated toxin does not revert to toxicity. If the activity of enhancing immunity is confirmed to be sufficiently high, then the preparation of attenuated toxin is completed. The chemical and physical treatments for attenuation can be properly combined [for attenuation].

The paragraph at page 22, lines 15 to 26 was changed by the replacement paragraph as follows:

Residual toxic activity can be assayed by any conventional method (see, for example, "Handbook for vaccination," Ed. Research Association, National Institute of Health of Japan, Maruzen, 1994; "Protein toxin," 1st and 2nd volumes, Ed., R. Tamura et al., Kodansha Scientific, 1972, etc.). The methods can be categorized as follows: enzyme activity assays, physiological-response assays using animal cells, physiological-response assays using animals, and evaluation methods utilizing survival and death of experimental animals, etc. The specific assay method to be used depends on the type of toxin. The following examples describe representative [indexes] activities as marker for selecting such a method. Specific procedures for these assays are known. The comparison of toxic activity is not limited to these indexes, but as the matter of course the comparison of toxin activities before and after the attenuation should be carried out using the same index.

The paragraph at page 33, line 28 to page 34, line 10 was changed by the replacement paragraph as follows:

A cholera toxin-producing bacterium (*Vibrio chorela*; Inaba type 569B strain) was cultured in a semi-synthetic casamino acid medium (liquid medium containing glucose) designed by Finkelstein et al., at 30°C for 20 hours. After the culture was completed, the culture supernatant was subjected to ultrafiltration, using a filter having pores that the cholera toxin molecule (molecular weight; 86,000 Da) could freely go through. A small amount of aluminum hydroxide gel was added to the filtrate. The gel was allowed to adsorb the toxin

and then was collected by centrifugation. The toxin was eluted from the gel with 10% phosphate-10% citrate (pH 7.6), while incubating at 30 to 35°C. The eluted liquid was dialyzed against an aqueous solution containing 0.1 M citrate. Then, the solution was loaded onto a column of DEAE-Sephadex and the toxin was eluted with phosphate buffer containing 0.1 to 0.2 M saline (hereafter abbreviated as PBS). Subsequently, the sample was loaded onto a column of Sephadex G-75 and the toxin was eluted with PBS. The resulting sample was subjected to gel electrophoresis. [Only the] The presence of the cholera toxin as a single band [was detected]. The purity was about 95%. The yield only was detected was 250 mg/100L culture [liquid] broth.

The paragraph at page 34, line 13 to 31 was changed by the replacement paragraph as follows:

The purified cholera toxin obtained in Example 1 was dissolved in 0.01 M PBS (pH 7-8). 0.05 M lysine was added to the solution. Then , to 6 aliquots of the solution formalin was added dropwise [to each aliquot of the solution] at [a] final [concentration] concentrations of 0.1, 0.3, 0.5, 0.6, 0.8, or 1.0%. The resulting solutions were incubated at 30 to 40°C for 7 to 96 days. Samples were [taken] withdrawn during the incubation. Immediately afterward, the collected samples were dialyzed against 20 times as much volume of PBS to remove formalin. The dialyzed solution was then sterilized by filtration to give attenuated cholera toxin preparations. The time course of the activity change was investigated by using a part of the samples collected during the treatment. Figure 1 shows [an example] a typical time course where the sample collected on the twelfth day of the formalin treatment was assayed by the method for measuring the residual toxic activity, utilizing as [an index the binding ability to ganglioside GM1, which is a receptor for] a marker the ability to bind cholera toxin B subunit (a subunit having the binding activity to target cells of toxin; A subunit is responsible for the toxic activity) to ganglioside GM1, which is its receptor. The result shows that the binding ability of the attenuated toxin to ganglioside GM1 is reduced to about 1/15 and 1/100 of the natural one, when the toxin was treated with 0.3% and 0.5% formalin for 12 days, respectively.

The paragraph at page 34, line 32 to page 35 line 9 was changed by the replacement paragraph as follows:

The Y-1 cell [morphologic] morphological transformation test was utilized to assay the residual toxic activity of a variety of samples that were subjected to attenuation treatment under various conditions. Part of the result is indicated in Table 2. The result shows that the residual activity is reduced by a factor of about 1/1780 to about 1/114000 when the toxin was treated with 0.3% and 0.5% formalin for 12 to 96 days. Acute toxicity test was conducted in mice by using the attenuated cholera toxin prepared above. The attenuated cholera toxin, indicated in Table 2, in which the residual toxic activity was 1/7000 of the original activity or lower, did not show toxicity when administered intraperitoneally at a dose of 30 mg/kg. Accordingly, the toxic activity can be reduced to one-two thousandth or lower (using acute toxicity in mouse as [an index] marker) by treating the toxin with 0.3% formalin at 35°C for 12 days or more.

The paragraph at page 35, lines 12 to 14 was changed by the replacement paragraph as follows:

Residual toxic activity of formalin-treated cholera toxin (according to the result obtained by Y-1 cell [morphologic] morphological transformation test).

The paragraph at page 35, line 18 to page 36, line 11 was changed by the replacement paragraph as follows:

HA antigen prepared from influenza virus PR8 strain (A/Puerto Rico/8/34, H1N1 type) that had been [habituated] adapted to mouse was used as an antigen for [vaccination] the vaccine. Attenuated cholera toxin was used as the adjuvant. The residual toxic activity of attenuated cholera toxin used was 1/1780 to 1/114000 that of the original activity (according to Y-1 cell [morphologic] morphological transformation test). Five Balb/c mice (6-weeks-old, [females] female) were used for each group in the test. The mice were anesthetized with sodium amobarbital, which was administered into the peritoneal cavity. 10 μl of PBS containing 1 μg vaccine antigen and 1 μg adjuvant was given dropwise to either nasal cavity of the mouse for intranasal immunization. Four weeks later, the secondary immunization was carried out in the same manner. Two weeks after the secondary immunization, sera and nasal washes were collected from the mice.

The paragraph at page 36, line 12 to page 37, line 2 was changed by the replacement paragraph as follows:

The titer of anti-influenza virus antibody in the serum was determined based on the HI antibody titer. After bloodletting, the nasal washes were collected from the mice by perfusing the right and left nasal cavities with 1 ml of PBS containing 0.1% bovine serum albumin (BSA). The quantities of anti-HA-IgA antibody in the nasal washes and anti-HA-IgG antibody in the sera were determined by enzyme immunoassay (ELISA). Prior to the assay for anti-HA-IgA, each well of EIA plate was treated with 50 μl of HA vaccine (5 μg/ml) suspended in a coating buffer. The plate was allowed to stand [still] for the coating at room temperature for 2 hours. The plate was then washed with PBS containing Tween-20 (hereinafter abbreviated as PBS-Tween). Subsequently, each well was coated with 100 μl of PBS containing 1% BSA and 0.1% NaN_3 to prevent [unspecific] <u>non-specific</u> reactions. The plate was allowed to stand [still] at 4°C overnight, and then washed with PBS-Tween. A 100-μl aliquot of adequately diluted nasal [wash] <u>washes</u> sample was added to each well. After several hours, the reaction solution was discarded and the well was washed with PBS-Tween. Subsequently, 100 μl of alkaline phosphatase-labeled goat anti-mouse IgA α chain-specific antibody (or alkaline phosphatase-labeled goat anti-mouse IgG antibody) diluted with PBS containing 1% BSA and 0.1% NaN_3 was dispensed into the respective wells. The plate was allowed to stand [still] at room temperature for an hour and then washed. Finally, p-nitrophenyl phosphate (1 mg/ml; Sigma Co.) dissolved in 10% diethanol amine buffer (pH 9.8) was added to each well for color development. The plate was allowed to stand [still] at room temperature for 20 to 30 minutes, and then the coloring reaction was monitored at O.D. (405 nm) in a plate reader.

The paragraph at page 37, lines 3 to 12 was changed by the replacement paragraph as follows:

Figure 2 shows the influence of an influenza vaccine, comprising attenuated cholera toxin prepared in Example 2 as the adjuvant, on the production of anti-influenza HA IgA antibody by the secondary response in the nasal [wash] <u>washes</u>. When the adjuvant-free vaccine was administered intranasally, the titer of anti-HA-IgA antibody was low. On the other hand, it was observed that the titer of anti-HA-IgA antibody in the nasal [wash] <u>washes</u> was markedly elevated in the group [subjected to] <u>given</u> primary and secondary [inoculation

of] vaccine containing attenuated cholera toxin. The titer of antibody is comparable to that of the control in which the same dose of natural cholera toxin was used.

The paragraph at page 37, lines 13 to 20 was changed by the replacement paragraph as follows:

Figure 3 shows the influence on the production of anti-HA IgG antibody in the serum in the above-described test. The attenuated toxin elevated approximately 15 times the titer of HA antibody in the serum as compared with that with the adjuvant-free vaccine. However, the antibody titer was about half of that with natural cholera toxin used as a control. These results show that the attenuated cholera toxin is useful as the adjuvant for [vaccination] vaccine, for example, when the local immunity is required to be enhanced.

The paragraph at page 37, line 23 to page 38, line 6 was changed by the replacement paragraph as follows:

A variety of attenuated cholera toxins, of which residual toxic activities were all [at least] less than one-two thousandth of that of the natural one, were used as the adjuvants. The same experiment as in Example 2 was repeated several times to assay the titer of anti-HA-IgA in the nasal washes and titer of IgG antibody in the blood of mice. The antibody titers determined in each assay were converted to relative values to those observed when the same amounts of natural cholera toxin were use as positive controls. The relative value is indicated as the ordinate axis and the attenuation rate of the toxin (relative residual activity to the natural one) was indicated as the abscissa axis (Figure 4). It is obvious that the majority of attenuated toxins, of which residual toxic activities are [at least] less than one-two thousandth that of the natural one (one-two thousandth = $4^{-5.46}$), exhibit high levels of antibody production-enhancing activity comparable to that of the same amount of natural cholera toxin. In particular, it was verified that the attenuated toxins, of which residual toxic activities are reduced to less than one-two thousandth that of the natural one, show comparable or higher efficacy [relative] as compared to that of original toxin on intranasal administration in regard to the enhancement of [mucous membrane] antigen-specific mucosal IgA formation [specific to the antigen by intranasal inoculation].

The paragraph at page 38, lines 9 to 22 was changed by the replacement paragraph as follows:

A cholera toxin attenuated by the formalin treatment (having a residual toxic activity of about 1/1048000 that of the natural one in Y-1 cell [morphologic] <u>morphological</u> transformation test) and *E. coli* heat-labile toxin B subunit (untreated), which toxic activity had previously been confirmed to be weak, [was] <u>were</u> used as the adjuvant together. This experiment was carried out in the same manner as in Example 3. The cellular immune response to influenza virus was evaluated by the mouse foot [swelling] pad test. The result is shown in Figure 5. When 1 μg of *E. coli* heat-labile toxin B subunit and 0.1 μg or 0.01 μg of attenuated cholera toxin were used together, the adjuvant enhanced immune response evidently much higher than that exhibited with the same amount of attenuated cholera toxin alone. The result shows that the dose of attenuated toxin can be further reduced by the method in which the attenuated toxin is used together with another adjuvant.

The paragraph at page 38, line 26 to page 39, line 10 was changed by the replacement paragraph as follows:

Diphtheria toxin and pertussis toxin was prepared according to a method described by the Kitasato Institute for producing diphtheria vaccine and pertussis vaccine ("Textbook of techniques for vaccine production," Kitasato Institute, 1986). Each toxin was attenuated in the same manner as shown in Example 2 and was adsorbed on aluminum gel and used in the subsequent experiments. In addition, commercial Staphylococcal α toxin (Sigma Co.) and enteritis vibrio thermostable toxin (Sigma Co.) were purchased and attenuated in the same manner as described in Example 2. The toxins were used without further modification. A recombinant mutant LTR(7)K (lysine residue is substituted for arginine residue at amino acid position 7 from the N terminus) of *E. coli* heat-labile toxin was prepared by a method described by Komase et al. (K. Komase et al., Vaccine 16, 248-254, 1998). Thus five types of attenuated toxins were obtained. The effect of enhancing immunity [associated with] <u>of</u> influenza vaccine was tested according to the same method as described in Example 3, except that the attenuated toxins prepared above were used at appropriate concentrations instead of the attenuated cholera toxin in Example 3. The result is shown in Table 3, where relative value of residual toxic activity of attenuated toxin (ratio of toxin attenuated versus the natural one) is also indicated.

The paragraph at page 39, lines 11 to 14 was changed by the replacement paragraph as follows:

The result shows that both adjuvants of the invention, whether the natural toxin or the recombinant mutant toxin, exhibit activity of enhancing immunity [associated with] of influenza [vaccination] vaccine administered by the intranasal route.

The paragraph at page 41, lines 1 to 15 was changed by the replacement paragraph as follows:

Vaccine prepared as above containing pertussis-diphtheria-tetanus combined vaccine and attenuated toxin was inoculated to mice of each group. The same amount of the vaccine was further given to each mouse for supplementary inoculation after 4 weeks. After 2 weeks following the last immunization antibody production was evaluated. When vaccine alone was inoculated into mice, the titers (international unit) of anti-pertussis toxin antibody, anti-diphtheria toxin antibody, and anti-tetanus toxin antibody were 2.0 units or lower, 2.0 units or lower, and 1.5 units or lower, respectively. On the other hand, the other group subjected to vaccination with attenuated toxin showed respective titers of 88.6, 61.8, and 75.5 units. The test result indicates that, in the group subjected to vaccination with attenuated toxin, the amount of produced antibodies against the respective antigens are larger than those in mice inoculated with the adjuvant-free vaccines.

The paragraph at page 42, lines 6 to 12 was changed by the replacement paragraph as follows:

A tetanus antibody assay kit "KAKETSUKEN" was used for assaying antibody titer in the blood. The assay result for the titer of antibody against tetanus toxin is shown in Table 4. After seven weeks, the antibody titer was found to be higher in the group [subjected to] given vaccine [innoculation] with attenuated cholera toxin than that observed in the group [subjected to] given vaccine [innoculation] without attenuated cholera toxin (control).

The paragraph at page 43, lines 13 to 14 was changed by the replacement paragraph as follows:

Example 10 - Pharmaceutical preparation containing [vaccine against] Japanese encephalitis [virus] vaccine and attenuated cholera toxin (injection):

The paragraph at page 44, lines 16 to 25 was changed by the replacement paragraph as follows:

The antibody titer in mouse blood was assayed according to the same method as described in Example 11 except that the attenuated pertussis toxin (the same as prepared in Example 6; having a toxic activity of about 1/100000 that of the natural one when observed by CHO cell [morphologic] morphological transformation test) was used instead of the attenuated cholera toxin used in Example 11. The ELISA antibody titer was 0.19 for measles or 0.070 for rubella in mice subjected to the inoculation of the adjuvant-free vaccine, and the titer was 0.32 for measles or 0.16 for rubella with attenuated pertussis toxin-containing vaccine.

The paragraph at page 44, line 27 to page 45, line 4 was changed by the replacement paragraph as follows:

Example 13 - Pharmaceutical preparation containing rotavirus vaccine and attenuated recombinant E. coli heat-labile toxin (oral [inoculum] vaccine, and nasal drop):

Rotavirus vaccine was mixed with a fraction [of] containing attenuated recombinant LTR(7)K (having a toxic activity of about 1/260000 that of the natural one; the same as in Example 6) that had been dissolved in PBS and sterilized by filtration. 20 μl of the solution contained rotavirus vaccine, of which amount corresponded to 3.5 μg of virus particles, as well as 10 μg attenuated recombinant LTR(7)K. A preservative (0.01% thimerosal) as well as a stabilizer (0.2% porcine gelatin) was added to the solution. The solution was dispensed into appropriate containers, and was used as the oral inoculum or nasal drop of rotavirus vaccine-attenuated LT toxin. The preparation was stored at a temperature of less than 10°C in a cool and dark place.

The paragraph at page 45, lines 21 to 24 was changed by the replacement paragraph as follows:

3. The adjuvant of the invention exhibits a comparable level of [immuno-enhancing] <u>immuno-potentiating</u> activity to that of the natural one at the same dosage, even when the residual toxic activity has been reduced to an undetectable level.

The paragraph at page 45, line 31 to page 46, line 9 was changed by the replacement paragraph as follows:

Thus, vaccine preparations containing attenuated toxin in accordance with the present invention are useful as adjuvants with high safety. Further, vaccines using the adjuvant of the invention are not only highly safe but also are excellent vaccines having sufficient activities of enhancing immunity even when used by non-injection vaccination route, such as intranasal, percutaneous or oral [inoculation, by] <u>ones, in</u> which, [with any conventional methods] sufficient [immunological effects are] <u>immunity is</u> not <u>usually</u> expectable <u>with any conventional methods</u>. In addition, as seen in the Examples, the vaccines in accordance with the present invention are excellent in inducing local immunity, cellular immunity, and such which were problems difficult to overcome with publicly known adjuvants. Moreover, it is also possible to utilize natural toxins as safe adjuvants according to the present invention. Thus, because of the simplicity, the invention is easily applicable to the vaccine as compared with approaches of generating mutant toxins.